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PHYSIOLOGICAL AND MORPHOLOGICAL STATE OF THE SYMBIOTIC BACTERIA FROM LIGHT ORGANS OF PONYFISH

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ABSTRACT

Symbiotic, bioluminescent bacteria (*Photobacterium leiognathi*) within and directly removed from the light organs of freshly sacrificed Philippine and Japanese ponyfish (family Leiognathidae) were analyzed for light production, oxygen uptake, morphology, and density. Luminescence averaged 2.4×10^4 quanta $\cdot s^{-1} \cdot cell^{-1}$ for bacteria from 24 fish (6 species in 3 genera), more than 10 times the maximum luminescences of *P. leiognathi* grown in culture. Light production (depending on the *in vivo* quantum yield for luminescence, 0.1 to 1.0) accounted for 1.7 to 17% of the total oxygen utilized by bacteria from the light organ, substantially more than found for *P. leiognathi* in culture. Bacteria from the light organ were non-motile, non-flagellated coccobacilloid to short rod-shaped cells (1.6×3.2 μm), whereas in culture they showed motility and polar flagellation. *In situ* doubling time for the population of light organ bacteria was estimated to be approximately one day, or 20 to 30 times slower than in culture. Within the tubules of the light organ, the bacteria were solidly packed inside elongate, thinly-walled saccules, with one to 20 saccules tightly filling each light organ tubule. The saccules held the bacteria at a density (calculated from bacterial cell and saccule volumes) of approximately 1×10^{11} cells $\cdot ml^{-1}$, which is a density roughly 15 times greater than estimated from total light organ volume. These findings lead to a maximal-luminescence, minimal-growth bacterial model of this symbiosis.

INTRODUCTION

Members of more than 30 families of marine fish utilize luminous bacteria for light production. The fish house their species-specific symbiotic bacteria in highly structured, specialized light organs and utilize the bacterial light in a variety of ways (Herring and Morin, 1978; Nealson and Hastings, 1979; Hastings and Nealson, 1981). For one of these associations, the light organ symbiosis between ponyfish and *Photobacterium leiognathi,* the host fish are readily accessible and much behavioral and anatomical information on them has recently been obtained (McFall-Ngai, 1983a, b; McFall-Ngai and Dunlap, 1983, 1984; Dunlap and McFall-Ngai, 1984). These studies provide a background for examination of ponyfish light organ bacteria.

Ponyfish (family Leiognathidae) are a group of small, schooling fish abundant in coastal waters of the Indo-West Pacific (for references, see Pauly and Wade-Pauly, 1981). Ponyfish emit several different bioluminescent displays that may function in feeding, defense, and reproduction (Hastings, 1971; Herring and Morin, 1978; McFall-Ngai and Dunlap, 1983). The versatility in types of luminescent displays results from the control over light emission provided by muscular shutters and chromatophores

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Abbreviations: BASW, buffered artificial sea water; PHB, poly-β-hydroxybutyrate; SWC, sea water complete medium.

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that cover the light organ, from the internal location of the light organ, and from several unique anatomical adaptations of the gas bladder, body musculature, and skin of the fish (Haneda, 1940; McFall-Ngai, 1983a, b; McFall-Ngai and Dunlap, 1983, 1984; Dunlap and McFall-Ngai, 1984). The internal, poorly vascularized ponyfish light organ overlies the esophagus just anterior to the stomach and interfaces with the oxygen-rich gas bladder across a thin, window-like membrane that is permeable to oxygen (McFall-Ngai, 1983a, b). Within the light organ, the dense, pure culture of *P. leiognathi* (Boisvert *et al.*, 1967; Hastings and Mitchell, 1971; Reichelt *et al.*, 1977; Jayabalan *et al.*, 1978) is held in numerous fine-diameter tubules that coalesce and empty into the esophagus via small ducts, thus allowing the bacteria to pass into the gut of the host (Harms, 1928; Haneda, 1940, 1950; Ahrens, 1965; Bassot, 1975). A facultative symbiont, *P. leiognathi* survives free of the host's light organ in various other marine habitats (Hastings, and Nealson, 1981).

The behavioral value of luminescence, and the anatomical and physiological commitment of the fish to maintaining light organ bacteria, indicate the importance of bacterial light production to the fish. Consequently, given the fish's secondary control of the intensity and type of light emission by muscular shutters and chromatophores over the light organ, maximal luminescence would appear to be adaptive in this association. In this regard, alternative rapid-growth and nutrient-limitation models for the ponyfish symbiosis have been proposed that are consistent with high levels of luminescence (Nealson, 1979). However, essentially no information is available on the intensity of bacterial luminescence *in situ* or on the control of luminescence by the fish at the primary (physiological) level.

The present study addresses questions of the physiological and morphological state of symbiotic bacteria in the ponyfish light organ. Measurements of bacterial light production and oxygen uptake, as physiological parameters of bacterial activity in the functioning symbiosis, are reported. The morphology, spatial distribution, and population density of light organ bacteria from several different ponyfish are described and interpreted in light of a newly discovered structural feature of this symbiosis, the light organ saccule. The results indicate that ponyfish bacteria grow slowly but produce consistently and exceptionally bright luminescence in the light organ, possibly in response to both anatomical and physiological controls exerted by the host fish. The results also provide a basis for *in vitro* studies of bacterial luminescence and growth that may lead to a better understanding of the physiology of this symbiosis.

Materials and Methods

Collection and handling of fish

The fish examined in this study were identified from the descriptions by Munro (1964), Kühlmorgen-Hille (1974), James (1975), Abe (1976), Rau and Rau (1980), and Dunlap and McFall-Ngai (1984). Ponyfish captured live from Manila Bay, Luzon and Bais Bay, Negros Oriental, Philippine Islands (*Gazza achlamys, G. minuta, Leiognathus bindus, L. brevirostris, L. equulus, L. fasciatus, L. splendens,* and *Secutor insidiator*), and at Misaki, Honshu, Japan (*L. nuchalis*), were maintained in laboratory aquaria near the collection sites as described by McFall-Ngai and Dunlap (1983) or were transported live to laboratory facilities at the University of California, Los Angeles and maintained under similar conditions. For morphological examination of bacteria, light organs from freshly sacrificed specimens of these fish and from fresh specimens of *L. elongatus* and *L. rivulatus* from Suruga Bay and the Sagami Sea, Japan, were preserved in potassium-phosphate buffered 10% formalin, pH 7.0. Only healthy specimens of the fish, held less than one week (typically 1 to 2 days), were used for

physiological and morphological analysis of live light organ bacteria. Live fish were individually netted from the holding aquarium and were quickly sacrificed by severing the spinal cord. Light organs were aseptically dissected from the fish, and esophageal and light organ shutter tissues were dissected away. Dissection typically took less than three minutes from the time of sacrifice. Width (lateral dimension), height (dorsal to ventral dimension), and length (anterior to posterior dimension) of the light organ were multiplied to calculate its volume, a method that may slightly overestimate volume due to the inclusion of the small esophageal space and because the light organ is only very roughly cuboidal. Immediately after being measured, light organs used in analysis of bacterial luminescence, oxygen uptake, and density were homogenized thoroughly in a sterile 8 ml Ten Broeck tissue homogenizer with 2.4 ml of 40% buffered artificial sea water (40% BASW = 11.0 g NaCl, 0.4 g KCl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.8 g $MgSO_4 \cdot 7H_2O$ to one liter with distilled water, and with 25 m*M* Tris·HCl, pH 7.5).

Luminescence and oxygen uptake

Using a photomultiplier-photometer similar to that of Mitchell and Hastings (1971), light production by bacteria was measured on light organ homogenates (in 40% BASW) diluted 1:1 or 1:3 with 40% BASW to eliminate possible quenching of luminescence (1 ml total volume). Using 40% BASW, replicate luminescence intensity measurements of aliquots of light organ homogenates were the same within ±5% when taken in the first minute after homogenization of the light organ. Thereafter, and more rapidly with homogenization fluid of higher salt content, luminescence declined markedly regardless of the extent of aeration. Therefore, all measurements of luminescence reported here were taken within 15 s following homogenization of the light organ. Luminescence is expressed as quanta $\cdot s^{-1} \cdot cell^{-1}$. The photometer was calibrated with light standard #231, which emits 9.7×10^8 quanta $\cdot s^{-1}$ ($\pm 0.2 \times 10^8$) at 480 nm (prepared and calibrated by J. W. Hastings, Harvard University). In a preliminary report of this study (Dunlap, 1983), light standard 'J' was used, but was found to overestimate light levels approximately 10-fold compared to standard #231. Because of this discrepancy and the present lack of uniform light standards, the more conservative standard #231 was used in the present report. Standard #231, however, may under- or over-estimate absolute light levels by 2 to 3 fold (K. H. Nealson, Scripps Institute of Oceanography, pers. comm.).

Using a polarographic oxygen electrode apparatus (Rank Brothers, Cambridge, England) calibrated to air-saturated 40% BASW, oxygen uptake by bacteria was measured on aliquots of light organ homogenates. The rate of oxygen uptake was measured over a 5 to 10 min period, and the reaction chamber temperature was maintained at the corresponding fish's aquarium temperature (27°C to 31°C, depending on the experiment). For direct comparison with luminescence, which uses oxygen as a reaction substrate (for reaction see Hastings and Nealson, 1981), oxygen uptake is expressed as molecules $O_2 \cdot s^{-1} \cdot cell^{-1}$. In certain cases, by suspending the organ in 40% BASW in the oxygen electrode reaction chamber, oxygen uptake was determined for intact light organs. Subsequently, those light organs were homogenized and handled as described above, and luminescence measurements were made. Oxygen uptake rates for bacteria released from the light organ directly into 40% BASW and for bacteria in association with tissue dissected from the light organ were essentially identical, which indicates that light organ tissue did not contribute measureably to oxygen uptake.

Morphology and population density of symbiotic bacteria

Scanning electron microscopy (SEM) was performed on formalin-preserved light organs that had been fixed for four hours in 2% glutaraldehyde (0.05 *M* Na-cacodylate buffer, pH 7.0). After three washes with buffer, the organs were post-fixed in 2% osmium tetra-oxide (0.05 *M* Na-cacodylate buffer, pH 7.0), again washed three times with buffer, and then dried through an ethanol series (10% to 100% ethanol). The light organs were then critical-point dried by carbon dioxide exchange in a Tousimis Samdri PVT-3 critical point dryer and sputter-coated with gold-palladium (50:50) in a Technics Hummer 1 sputter coater. The material was examined with an Etec Autoscan scanning electron microscope operated at 10 kV.

Using a phase contrast Zeiss microscope calibrated with ocular and stage micrometers, both freshly dissected and preserved light organs were examined for bacterial cell shape and dimensions, the number of bacteria visibly in division and the dimensions of light organ saccules. Flagellar staining of bacteria removed from freshly dissected light organs and, for positive controls, of bacteria cultured overnight on sea water complete (SWC; Nealson, 1978) agar and broth were carried out using the Leifson method (Doetsch, 1981).

For starvation studies, specimens of *L. splendens* were held in 30-gallon sea water aquaria with aeration, but without food for three weeks. Beginning two days after capture, two fish were sacrificed every two days and their opened light organs were examined visually for luminescence in a well-darkened room. To confirm the presence of symbiotic bacteria, contents of the light organs were streaked on SWC agar and examined for luminescent bacterial growth the following day.

Counts of viable bacteria, to estimate the total number of bacteria present in a light organ and their density (bacteria $\cdot$ ml^{-1} of light organ volume), were made by spread-plating 0.1 ml of the appropriate serial dilutions of light organ homogenates on duplicate SWC agar plates. Platings were completed within 10 min of homogenization of the light organ. Colonies were counted after 12 to 16 hours of incubation at 28°C; more than 99.9% were luminescent and uniform in colony morphology (>1500 colonies examined per plating), although in many instances the intensity of luminescence ranged from dim to very bright for colonies on the same SWC agar plate. A few randomly picked colonies from platings of homogenates from several different light organs were retained for taxonomic identification; all were confirmed as *P. leiognathi* according to the methodology of Reichelt and Baumann (1973) and Baumann and Baumann (1981), as previously reported for ponyfish light organ bacteria (Reichelt *et al.*, 1977). Using a Petroff-Hauser counting chamber, total direct counts of bacteria from light organ homogenates were found to be equal to viable counts (n = 3), indicating that 100% of the light organ bacteria are viable. Stomachs and intestines of several fish specimens were also removed aseptically, homogenized, and plated as described above. Freshly voided fecal pellets were handled in a similar way.

Results

Luminescence and oxygen uptake by symbiotic bacteria

Ponyfish bacteria directly removed from the light organ were consistently very brightly luminescent. For bacteria from 24 fish (in three genera and six species), the mean light intensity was 2.4×10^4 quanta $\cdot$ s^{-1} $\cdot$ cell^{-1} ($\pm$S.D.: 1.2×10^4; range: 0.9×10^4 to 4.7×10^4). This value was at least 10 times greater than maximum levels of luminescence produced by ponyfish bacteria in culture ($<10^3$ quanta $\cdot$ s^{-1} $\cdot$ cell^{-1})

and by bacteria taken directly from the light organ of the apogonid fish, *Siphamia cephalotes* (2.3×10^3 quanta $\cdot s^{-1} \cdot cell^{-1}$; Fitzgerald, 1978), which have been tentatively identified also as *P. leiognathi* (Reichelt and Baumann, 1973; Fitzgerald, 1978). Similar levels of light production (10^4 to 10^5 quanta $\cdot s^{-1} \cdot cell^{-1}$) have been reported for various bacteria in culture (Nakamura and Matsuda, 1971; Hastings and Nealson, 1977; Karl and Nealson, 1980), but meaningful comparisons between studies must await the availability of uniform light standards.

For 10 of these 24 ponyfish, both the luminescence and oxygen uptake rates of the symbiotic bacteria were determined. Luminescence showed a positive correlation with oxygen uptake (correlation coefficient, r = 0.84), and the levels of luminescence and oxygen uptake appeared to relate to the species of fish housing the bacteria (Fig. 1). Using a range of 0.1 to 1.0 for the as yet unknown *in vivo* quantum yield of luminescence (for discussion see Hastings and Nealson, 1977; Karl and Nealson, 1980), luminescence accounted for 1.7 to 17% of the total oxygen taken up by the bacteria (Table I). However, oxygen uptake by bacteria within intact light organs was half that of bacteria in light organ homogenates (Table II), so the percentage of oxygen consumed due to luminescence might be as high as 3.4 to 34%. In contrast, when grown in culture to peak luminescence, *P. leiognathi* consumes substantially less oxygen in light production, 0.02 to 0.2% (manuscript in prep.). Thus, the activity of ponyfish light organ bacteria *in situ* is characterized both by brighter luminescence and by a higher proportion of oxygen consumed in light production compared to *P. leiognathi* in culture. Earlier reports of oxygen consumption in luminescence by maximally luminescent bacteria in culture give generally similar percentages (2 to 53%: Eymers and van Schouwenberg, 1937; Harvey, 1952; Watanabe *et al.*, 1975; Karl and Nealson, 1980).

Morphology and density of symbiotic bacteria in the light organ

The symbiotic bacteria reside at high density inside narrow-diameter light organ tubules (Fig. 2), of which there are 150 to 200 (Bassot, 1975). External diameters of

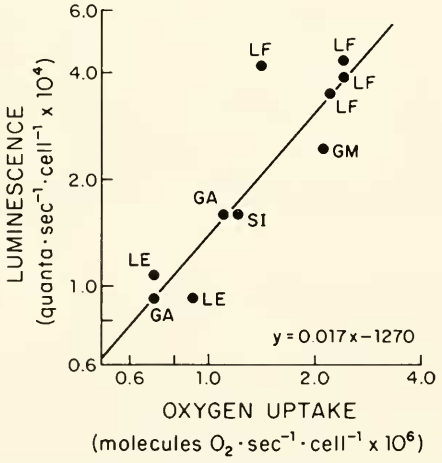

FIGURE 1. Plot of oxygen uptake *versus* light production for bacteria released from light organs of 10 ponyfishes. Fish species designations: GA, *Gazza achlamys;* GM, *G. minuta;* LE, *Leiognathus equulus;* LF, *L. fasciatus;* SI, *Secutor insidiator.*

TABLE I

Activity of symbiotic bacteria taken directly from ponyfish light organs

	Luminescence (quanta · s^{-1} · $cell^{-1}$) × 10^4	Oxygen uptake (O_2 · s^{-1} · $cell^{-1}$) × 10^6	O_2 consumed in luminescence (% of total)
$\bar{X}$[a]	2.5	1.5	1.7–17[b]
(±S.D.)	(1.4)	(0.7)	
range	0.9 to 4.4	0.7 to 2.4	

[a] n = 10 fish (see also Fig. 1).

[b] Lower and upper limits. *In vivo* quantum yield for luminescence is unknown but probably falls between 0.1 and 1.0 (see Hastings and Nealson, 1977; Karl and Nealson, 1980).

tubules in freshly dissected light organs ranged from 25 to 70 μm, a size similar to the 35 μm diameter reported by Bassot (1975) for tubules in light organs prepared for transmission electron microscopy (TEM). However, much variation in tubule wall thickness and tubule lumen diameter was noted for the different species of fish.

Bacteria taken directly from light organs of freshly sacrificed ponyfish and examined with phase contrast microscopy were, in general, uniformly shaped coccobacilloid to short rod-shaped cells averaging 1.6 × 3.2 μm. Among the fish examined, only the bacteria of *L. splendens* and *L. elongatus* varied much from the regular coccobacilloid shape. A high proportion of *L. splendens* bacteria (from light organs of seven juvenile specimens) were spherical, cube-shaped, pear-shaped, or blunt-ended, with distortion possibly due to tight cell packing during growth in the light organ tubules. Similar distortions of bacterial cell shape were noted by Bassot (1975). Bacteria of *L. elongatus,* which have only recently been brought into culture (Dunlap, 1984), were distinctly more elongate than those of other ponyfish (as also shown by Haneda and Tsuji, 1976). Bassot (1975) also noted the occasional presence of apparently lysed bacteria and 'ghost membranes' in the light organ of *L. equulus.* In the present study, such forms were seen only once, among otherwise normal-looking bacteria of one specimen of *L. fasciatus.* In this case, the 'ghost' bacteria represented approximately 60% of the bacteria from one tubule, whereas bacteria from other tubules of the light organ of this fish appeared normal.

As judged by phase contrast examination of wet mounts made from freshly dissected light organs (from 25 fish and several thousand bacteria examined per light organ), ponyfish bacteria lacked visible granules of poly-β-hydroxybutyrate (PHB) (also shown to be absent in TEM micrographs by Bassot, 1975; see Nealson, 1979) and in all

TABLE II

Rates of oxygen uptake of bacteria in intact and homogenized ponyfish light organs

Source of light organ	Intact organ (O_2 · s^{-1} · $cell^{-1}$ × 10^6)	Homogenized organ (O_2 · s^{-1} · $cell^{-1}$ × 10^6)	Intact:homogenized
Leiognathus equulus	0.5	0.9	0.55:1
L. equulus	0.3	0.7	0.43:1
Gazza achlamys	0.3	0.7	0.43:1
G. achlamys	0.5	1.0	0.50:1
			$\bar{X}$ 0.48:1

FIGURE 2. SEM of ponyfish (*Gazza minuta*) light organ tubules, which house the bacterial symbionts (*Photobacterium leiognathi*). Light organ saccules, apparently destroyed in preparation of the light organ for SEM, are not visible. Note the scarcity of capillaries (as noted by Bassot, 1975) and the density and uniformity of shape of the bacterial cells in the tubules. Size bar = 25 μm.

cases were non-motile. Using the Leifson method (Doetsch, 1981), staining for flagella gave negative results, whereas controls grown overnight in SWC broth and on SWC agar showed active motility and polar flagellation (as described for *P. leiognathi* in culture; Reichelt and Baumann, 1973). Motility of bacteria in light organ homogenates developed after 6 to 12 hours at 24°C.

Four to five percent of the 1200 bacteria examined from one fish (*G. minuta*) were visibly in division. Using a regression formula that relates frequency of dividing bacterial cells (FDC) to growth rate of a natural population, $\ln \mu = 0.299 \text{ FDC} - 4.961$ (Newell and Christian, 1981), the growth rate of the bacteria was estimated to be 0.03 h^{-1}. In culture, growth rates of 0.60 h^{-1} or higher are common for *P. leiognathi*. Thus, the light organ bacteria *in situ* double slightly less often than once

per day, at least 20 times slower than in culture. Using different methods, Haygood *et al.* (1984) found comparably slow *in situ* growth rates for *V. fischeri* from the light organs of pinecone fish (family Monocentridae).

Viable, brightly luminescent bacteria were retained in the light organs of all unfed specimens of *L. splendens* up to the point of death by starvation and cannibalism (3 weeks). With respect to the digestive tract, roughly equal volumes of the stomachs and intestines of several freshly sacrificed specimens of *L. nuchalis* held approximately equal numbers of luminous bacteria (2 to 3×10^5 cells). Additionally, fecal pellets released from various live, freshly captured ponyfish were brightly luminescent. 70 to 100% of the bacterial colonies that formed on SWC agar from homogenates of the stomach, intestines, and fecal pellets were luminescent and appeared identical in intensity and color of luminescence and in colony morphology to colonies formed by the light organ bacteria from the same fish. Although these observations require better quantification and taxonomic confirmation, they indicate that *P. leiognathi* may grow slowly in the light organ and, once released from the light organ, may pass through the gut and survive digestion in substantial numbers.

Estimates were made of the light organ volumes of 16 ponyfish and the total number and density of symbiotic bacteria in the organs (Table III). Although the fish specimens were of different species and sizes (which can influence light organ volume; McFall-Ngai and Dunlap, 1984), light organ volume was found to increase in parallel with the standard length of the fish ($r = 0.88$). The calculated total number of bacteria per light organ (Table III) increased as light organ volume increased ($r = 0.73$) and was comparable to values reported for ponyfish from New Guinea (Hastings and Mitchell, 1971) and from India (Jayabalan *et al.*, 1978). In addition, the density of bacteria (bacteria $\cdot$ ml^{-1}) in these light organs (Table III) was similar to that reported for other ponyfish (Hastings and Mitchell, 1971), for pinecone fish (Ruby and Nealson, 1976), for flashlight fish (family Anomalopidae) (Herring and Morin, 1978), and for *P. leiognathi* grown in SWC broth culture to stationary phase (4 to 5×10^9 cells $\cdot$ ml^{-1}). Using a mean bacterial cell volume of 6 to 7 μm^3 (6 to 7×10^{-9} μl), ponyfish bacteria of the present study were calculated to take up 4 to 5% of the volume of the light organ. This estimate may be low due to a possible overestimation of light organ volume (see Materials and Methods). Using a comparison between light organ wet weight and a packed-cell wet weight count of luminous bacteria of 5×10^{10} cells/g, Hastings and Mitchell (1971) estimated that ponyfish bacteria make up 10 to 25% of the mass of the light organ.

TABLE III

Quantification of bacteria in light organs of ponyfish[a]

	Standard length of fish (mm)	Light organ volume (ml)	Bacteria per light organ ($\times 10^8$)	Bacteria per ml in light organ ($\times 10^9$)	Bacteria per ml in saccules[b]
$\bar{X}$	79	0.036	2.5	6.4	1×10^{11}
(±S.D.)	(15)	(0.013)	(2.2)	(4.4)	
range	49–98	0.013–0.056	0.13–8.1	1.0–15.0	

[a] n = 16 fish: *Leiognathus equulus*, 3; *L. fasciatus*, 7; *L. splendens*, 1; *Gazza achlamys*, 3; *G. minuta*, 1; *Secutor insidiator*, 1. See text for details.

[b] Calculated on the basis of bacterial cell and saccule volume and with the assumption that interstitial space between bacteria accounts for 26 to 40% of saccule volume.

However, instead of being freely dispersed in light organ tubules as is suggested by their appearance in SEM (Fig. 2) and TEM micrographs (Bassot, 1975), the bacterial symbionts were found to be tightly packed within numerous long, thin, bag-like structures (Figs. 3, 4) that have not previously been described for ponyfish. Apparently similar, spherically shaped structures have been reported for light organs of the macrourid fish, *Physiculus japonicus, Coelorhyncous kishinouyei,* and *Malacocephalus laevis* (Kishitani, 1930; Yasaki and Haneda, 1935; Haneda, 1938), which harbor *P. phosphoreum* (Ruby and Morin, 1978; Herring and Morin, 1978). These structures were called 'sackchen' by Kishitani (1930), so the term 'light organ saccule' is used here. In gently dissected ponyfish light organs, all bacteria were contained within light organ saccules, and light organs of all fish examined (14 individuals from 6 species in 2 genera) exhibited these saccules. The saccules were transparent, apparently acellular (Fig. 3A), and were themselves tightly fitted into the tubules. Depending on tubule diameter, tubules contained from 1 to 20 saccules in tight bundles (Fig. 3B), which conformed to the dimensions of the internal diameter of the light organ tubules (20 μm for one saccule to 50 μm for a bundle of several saccules). Saccules appeared to run the entire length of the light organ tubule, at least 400 to 600 μm). Most saccules had a short extension at one end, which may be a secretion or attachment point (Fig. 4A). Due to the fragility of saccules, more precise quantification of their dimensions and number within light organ tubules was not possible. They were easily damaged during dissection and were distorted by pressure of the overlying cover slip during examination, which further revealed the tight packing of bacteria (Fig. 4B). Plate 3, figure 3 of Bassot (1975) shows what may be a saccule around the tightly massed bacteria within a tubule. Using comparisons between bacterial cell and saccule volumes, a saccule 20 μm in diameter by 600 μm in length was estimated to contain approximately 2×10^4 bacteria. Thus, to account for the total number of bacteria present in a light organ, approximately 1250 saccules of this size would be necessary. This number, which is 6 to 8 times larger than the number of light organ tubules cited by Bassot (1975), is consistent with the observation that many tubules hold more than one saccule.

Assuming that interstitial space between the tightly packed cells accounts for 26 to 40% of saccule volume, as for the packing of spheres (Sloane, 1984), the density of bacteria within a saccule was calculated to be approximately 1×10^{11} cells $\cdot$ ml^{-1} (Table III), a value 15 times higher than the density calculated on the basis of total light organ volume. This bacterial density within saccules is also much higher than the density estimated for bacteria of pinecone fish (Ruby and Nealson, 1976) and flashlight fish (Herring and Morin, 1978). However, no examination for saccules in the light organs of these or other bacterially bioluminescent fish (*e.g.,* apogonids, pempherids) except macrourids has been reported. Additionally, based on the estimated interstitial space within a saccule and the volume of the light organ taken up by bacteria (4 to 5%), the volume of fluid covering the bacteria within the saccules ('light organ fluid') of a typical light organ (0.036 ml, Table III) was calculated to be approximately 0.5 μl per light organ. The coccobacilloid shape of the bacteria may permit slightly closer packing than possible with spheres, so the volume of light organ fluid might be slightly less.

Discussion

This analysis of *P. leiognathi* within and directly removed from the ponyfish light organ provides insight into possible interactions between the host fish and symbiotic bacteria in the functional luminescent association. These interactions would appear

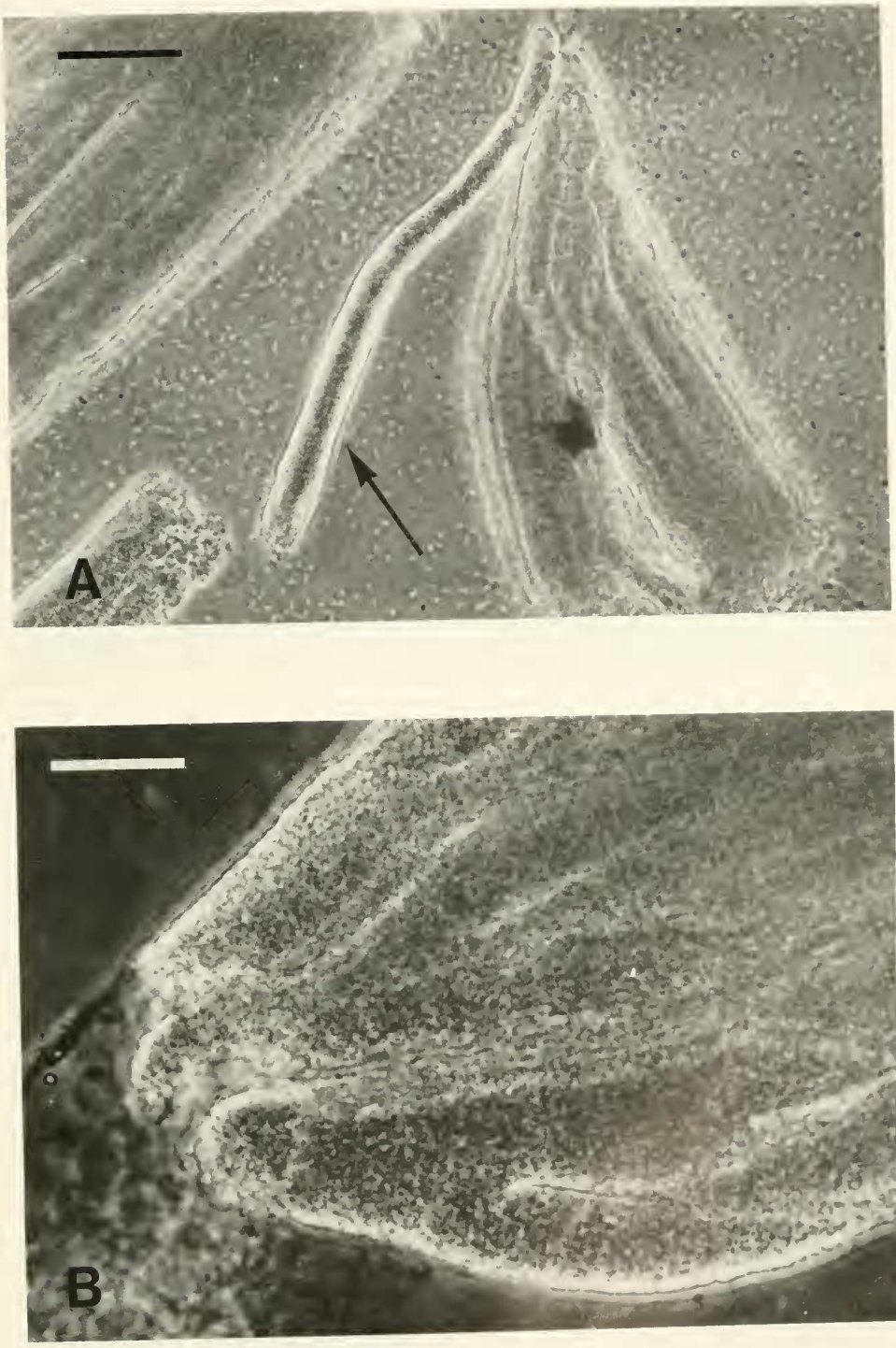

FIGURE 3. Ponyfish light organ saccules. (A) Bacterial symbionts are held inside elongate, thinly walled, transparent saccules (arrow), which are readily extruded from the light organ tubules (size bar = 75 μm); and (B) saccules, shown here extruded from the tubule, are present in bundles of up to 20 saccules per tubule (size bar = 20 μm). Note the solid masses of bacterial cells inside the saccules.

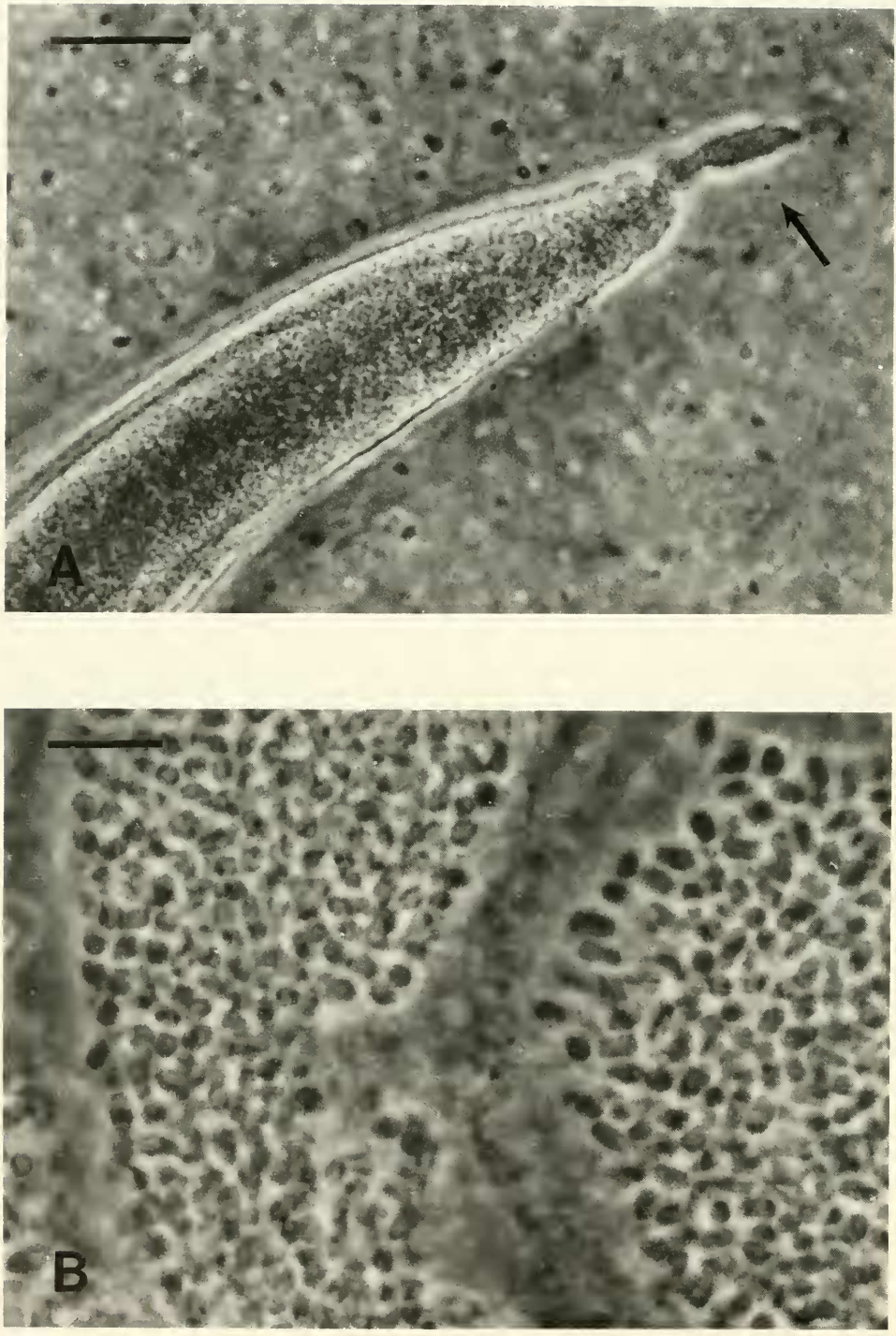

FIGURE 4. Ponyfish light organ saccules. (A) Each saccule bears a filament at one end (basal end?), possibly a site of attachment or secretion within the light organ tubule (size bar = 20 μm); and (B) the symbiotic bacteria occur in the saccule as tightly packed masses of coccobacilloid cells (size bar = 10 μm).

to derive from the controlling influences of micro-anatomical and as yet unknown physiological conditions of the light organ on bacterial luminescence and growth. Such control is reflected in the unusually bright luminescence and the higher percentage of oxygen consumed in luminescence by bacteria from the light organ, and in differences in their morphology and density within the light organ (Figs. 2–4), compared with in culture. The results of this study suggest a maximal-luminescence, minimal-growth model for bacterial activity in the ponyfish light organ and provide a basis for *in vitro* culture studies of the bacteria that may lead to a better understanding of the physiology of this symbiosis.

The fairly consistent and unusually high level of luminescence of bacteria from the light organ suggests that oxygen concentration in the light organ is sufficient for maximal induction and expression of the bacterial luminescent system. Ponyfish light organ bacteria appear to derive a substantial amount of their oxygen from the oxygen-rich gas bladder (McFall-Ngai, 1983b), which interfaces directly with the light organ across a thin oxygen-permeable membrane (McFall-Ngai, 1983a, b). In culture, oxygen concentration directly influences the luminescence of *P. leiognathi*, with higher levels of luciferase and brighter luminescence being produced when oxygen concentration is higher (Nealson and Hastings, 1977). Bacteria that produced more light took up more oxygen (Fig. 1), which suggests that a causal relationship might exist in the light organ between these two activities, with higher levels of available oxygen possibly leading to generally higher synthesis of luciferase and brighter cells. In this regard, the apparent trend for the bacteria of *L. fasciatus* and *G. minuta* to luminesce more brightly and take up more oxygen than the bacteria of the other fish (Fig. 1) could reflect species-based differences in oxygen concentration in the light organ.

At an oxygen concentration in the light organ sufficiently high for maximal luminescence, the symbiotic bacteria might exhibit rapid growth assuming other factors are not limiting; *P. leiognathi* grows rapidly in culture under such conditions (Nealson and Hastings, 1977). Rapid growth of the bacteria in the light organ could be a substantial energetic drain on the fish, one it might reduce by digesting the bacteria as they are released into the gut tract (Nealson, 1979). However, the apparently slow growth rate of bacteria in the light organ, their maintenance in the light organ during severe starvation of the fish, and their apparent ability to survive digestion are not consistent with this rapid-growth, digestion model. In contrast, these observations are consistent with an alternative, growth-limitation model in which the fish controls the growth of its light organ bacteria by restricting their supply of nutrients (Nealson, 1979) and consequently expends less energy in maintaining them.

The lower rate of oxygen uptake by bacteria in intact light organs compared to light organ homogenates (Table II) might indicate that the bacteria experience some oxygen limitation *in situ*. If so, the fish could be envisioned as controlling its bacteria by poising the oxygen concentration at a level that restricts growth but does not affect the expression or synthesis of luciferase. However, the available data indicating lower expression and synthesis of the luminescent system of *P. leiognathi* under low oxygen concentrations (Nealson and Hastings, 1977) are not consistent with this possibility. Alternatively, the removal of the light organ from the fish, and consequently from its normal oxygen delivery systems (gas bladder, circulatory system), may have created a surface-to-volume diffusion problem that accounts for this difference.

A newly discovered aspect of the micro-anatomy of the ponyfish light organ, the light organ saccule (Figs. 3, 4), may play a direct role in controlling luminescence by maximizing the density of bacteria. Light production by many other luminous bacteria has been demonstrated to be under the control of a density-dependent autoinduction system (Eberhard, 1972; Nealson, 1977; Ulitzur and Hastings, 1979; Rosson and

Nealson, 1981) and preliminary evidence is available for such a system in *P. leiognathi* isolated from ponyfish light organs (Dunlap, 1984). At the bacterial density found in light organ saccules, autoinducer concentration would presumably also be exceptionally high, thus maintaining the luminescent system of the bacteria in a maximally induced state. This form of control, coupled with an adequate level of oxygen for full induction and expression of the luminescent system, may partially or fully account for the observed high level of luminescence produced by the bacteria.

A second possible effect of light organ saccules may be to limit the flux of nutrients into the saccule lumen both by forming a diffusion barrier between the bacteria and host tissue and by maintaining a high bacterial density, which would keep the availability of nutrients low. That the bacteria of *L. splendens,* and of other ponyfishes (Bassot, 1975), are distorted in shape, due apparently to tight cell packing in the light organ during growth, emphasizes that the density of bacteria in the light organ is extremely high and distinguishes light organ growth conditions from those in culture on solid or liquid media. Saccules might also function to restrain the bacteria and allow nutrients to diffuse freely between them without permitting the bacteria to escape readily from the light organ tubules. The intense crowding of bacteria in the saccules may also account for the lack of motility, flagella, and poly-β-hydroxybutyrate (PHB) through density or nutrient-limitation effects. The shift to flagellated, motile cells some hours after escape from the light organ [and the development of PHB in culture (Reichelt and Baumann, 1973; Bassot, 1975; Nealson, 1979)] could involve release from these putative physical or nutritional constraints. Such possibilities are consistent with the nutrient-limitation model of this symbiosis proposed by Nealson (1979). The effects of nutrient limitation on light production, flagellation, and PHB deposition in these bacteria are, however, unknown, as are the type and fate of nutrients available.

Based on the measurements and observations of *P. leiognathi* presented here, the host fish is postulated to produce conditions under which the symbiotic bacteria luminesce maximally but grow at a minimal rate. The fish may evoke this response by presenting the bacteria with a growth-restrictive environment, possibly by limiting carbon directly (Nealson, 1979) or indirectly (high bacterial density in light organ saccules), or by iron-limitation (Haygood and Nealson, 1984). The apparently slow growth of the bacteria *in situ* and the sufficiently high oxygen concentration for maximal luminescence in the light organ implicate a form of growth restriction that acts at the level of the respiratory system. If control is manifested by such 'respiratory-restriction,' the bacteria might be responding adaptively by using the luminescent system as an alternative respiratory pathway to re-oxidize reduced coenzymes (as suggested for bacteria in culture by Hastings and Nealson, 1977, and Ulitzur *et al.,* 1981) and thus carry out maintenance metabolism and some growth. The very high level of luminescence produced by the bacteria and the high proportion of oxygen consumed in luminescence support this notion. Under these conditions, the fish would benefit doubly, first by minimizing the energetic cost of maintaining the bacteria and second by maximizing the physiological product (bioluminescence) that it obtains from them.

Although the nature of controls that could function to evoke a maximal-luminescence, minimal-growth response remain speculative at this time, the observations presented here should serve to stimulate comparisons between the physiological activity and morphological appearance of *P. leiognathi* in the ponyfish light organ and in culture. More specifically, the results of this study suggest that *in vitro* growth-restrictive culture conditions are likely to exist under which *P. leiognathi* will produce brighter luminescence than usual (*ca.* 10^4 quanta $\cdot s^{-1} \cdot cell^{-1}$). If these conditions exert their

control at the level of the respiratory system, oxygen would probably be taken up less rapidly, but proportionally more of it might be utilized for luminescence. As a consequence, *P. leiognathi* grown under such conditions might have reduced levels of functional cytochrome proteins, but would possibly be very rich in luciferase. Additionally, PHB and flagella might not be formed. Elucidation of the putative culture conditions evoking such responses from *P. leiognathi* may provide valuable insight into the nature of the controls used by the fish in this light organ symbiosis.

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